Review

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Optimization of biomass saccharification processes with experimental design tools for 2G ethanol production: a review

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Abstract: The current energy scenario has encouraged the replacement of carbon sources with renewable sources. Second-generation (2G) ethanol production from lignocellulosic material or agroindustrial biomass involves a three-step process consisting of pretreatment, saccharification and fermentation. Enzymatic saccharification is the most critical step for recovering fermentable sugars from biomass. Many factors such as pretreatment, enzyme load, pH, temperature, substrate and surfactant concentrations, and reaction time, among others, can affect biomass saccharification. In this sense, different design of experiment (DoE) tools together with response surface methodology (RSM) have been used as a viable strategy to determine how factors influence saccharification and to optimize this process. The strategy involves fewer experiments, less time and lower cost. This review summarizes how DoE and RSM tools have been used to optimize biomass saccharification. Moreover, an overview of the main experimental design tools used in this field is provided. © 2023 Society of Industrial Chemistry and John Wiley & Sons Ltd.

Key words: enzymatic saccharification; 2G ethanol; optimization; experimental design; response surface methodology

Introduction

The current world energy consumption is estimated at 5.42×10^{17} BTU, and this number is expected to rise by 50% until 2050.[1,2] In this scenario, replacing traditional carbon sources (non-renewable) with alternative ones has gained prominence worldwide,[1,3–5] so the use of renewable energy sources such as solar power, wind, water and plant biomass is expected to grow and to meet approximately 85% of the world demand by 2050.[1] Plant biomass has played a central role in this context: around 1.5×10^{10} tons of lignocellulosic waste are generated every year around the world.[3] According to a goal defined by COP21, these sources should be increasingly used to reduce

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the production of greenhouse gases like CO_2, NO_2 and CH_4 by up to 43%.[3,6–8]

Lignocellulose is mainly composed of cellulose, hemicellulose and lignin. Cellulose strains are bundled together to form cellulose fibrils *via* hydrogen bonding, whereas hemicellulose serves as a connection between lignin and cellulose.[1,4,9] Remarkably, cellulose is not only the most abundant fraction in lignocellulose, but also the fraction of greatest industrial interest.[1,10] Cellulose is a homopolymer composed of 10 000–140 000 cellobiose subunits (glucose dimers) linked to each other by β-1,4 linkages. The hydroxyl groups in the chain axial regions establish intra- (same molecule) and intermolecular (with other molecules) hydrogen bonds, forming highly compact and insoluble microfibrils. Together, these microfibrils form macrofibrils that resist chemical and biological agents.[10] The hemicellulose portion consists of polysaccharides with a highly variable configuration of hexose (such as glucose, galactose and mannose) and pentose (such as xylose and arabinose) monomers and uronic acid units (mainly 4-*O*-methyl-D-glucuronic acid).[1,10] Lignin involves cellulosic macrofibrils and is associated with different hemicellulosic matrix components, so it is the main physical barrier to physical, chemical and biological agents used in industrial processes and the portion that impacts lignocellulosic biomass recalcitrance the most. Recently, lignin has been the object of industrial interest for different purposes.[1,6,10]

Lignocellulosic biomass conversion to bioethanol requires several stages that include biomass pretreatment and fractionation, the production of suitable cellulolytic/hemicellulolytic enzymes, enzymatic pretreated biomass hydrolysis, fermentation of enzymatic hydrolysate by suitable microorganisms and downstream processing.[11] Pretreatment involves changing the cellulose–hemicellulose–lignin matrix structure[12] by removing lignin, partially or totally hydrolyzing hemicellulose, and reducing the cellulose crystalline fraction (relative to amorphous cellulose) and the degree of polymerization, to allow subsequent hydrolytic processes and sugar release to occur.[11,12] The lignocellulosic material pretreatment conditions depend on the type of biomass, which requires that process conditions for each biomass be studied and optimized.[13]

Saccharification of cellulose and hemicellulose polymers of pretreated biomass is the central step in 2G ethanol biorefineries.[13] Cellulases like endoglucanases, cellobiohydrolases, and β-glucosidases have been used for enzymatic pre-treated biomass hydrolysis.[12] Enzymatic hydrolysis of lignocellulosic biomass depends on enzyme-related and substrate-related factors. Therefore, factors such as temperature, saccharification time, pH, enzyme loading, substrate loading, addition of chemical agents like surfactants and mechanical agitation should be studied and optimized, to increase the concentration of sugars, and consequently hence ethanol production.[12] Biomass pre-treatment and enzymatic hydrolysis of recalcitrant biomass are among the major technical and economic impediments to the overall success of biorefineries.[14] Thus, alternatives such as simultaneous saccharification and co-fermentation have been proposed to diminish production costs. This strategy allows enzymatic hydrolysis and fermentation to be carried out in the same reactor, thereby avoiding substrate repression during cell metabolism, reducing contamination risks and preventing enzyme inhibition.[15] Given that many factors can be studied and optimized in the different strategies used to produce 2G bioethanol, some researchers have employed design of experiment (DoE) tools and response surface methodology (RSM) to achieve this goal.

Design of experiment tools comprise a smart approach that provides fast, efficient and economical strategies—these tools allow several factors and their interactions to be studied by means of fewer experiments than the one factor at a time approach.[16] Design of experiment tools are commonly used to study and to optimize process conditions or formulations during the development of new products and include screening designs [Plackett–Burman design (PB), fractional factorial design and Full Factorial Design (FFD)] and RSM, which encompasses central composite design (CCD) and Box–Behnken design (BBD). Screening designs help to discriminate the critical process variables by means of a reduced number of experiments; CCD and BBD are subsequently applied to infer the best levels of the previously determined significant factors, to optimize the process. Choosing which variables to study depends on the criteria and the researcher's knowledge about the evaluated system.[17–19]

Here, we analyze and discuss some papers on the application of DoE tools and RSM in biomass saccharification for 2G ethanol production published in the last 10 years. We show how these tools can be applied to study and to optimize the factors that influence biomass saccharification with a view to encouraging researchers to use these tools in future works in this area.

2G ethanol production in biorefineries

Agroindustrial biomass resulting of food harvest and processing is gaining researchers' interest for 2G ethanol production owing to its cellulose and hemicellulose content,

Table 1. Lignocellulosic composition of different biomass.

Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Apple bagasse	11.14	12.5	14.5	21
Barley straw	22.0–42	20.4–28	17.1–19.3	21,22
Bean straw	40.2	19.3	18.1	23
Coffee pulp	10.3	18.3	11.4	21
Corn straw	29.6–42.6	17.0–35.0	7.0–21.0	21,22
Elephant grass	15.2	14.0	10.3	21
Guinea grass	14.4	15.7	8.6	21
Grape stalk	14	11.7	23	23
Oat straw	37.6	23.34	12.85	22
Rice straw	32.0–47.0	16.0–28.0	5.5–24.0	22–25
Sisal fiber	64.9–78	10–25.4	8–11.7	26
Sorgum straw	32.4	27.0	7.0	22
Soybean straw	39.8	22.6	12.8	27
Sweet sorghum bagasse	42	23	14	28
Sugarcane bagasse	18.6–45.4	16.6–28.7	10.6–23.4	21,22
Wheat straw	33.0–45.0	19.0–32.0	8.0–28.0	22–29

which are sources fermentable sugars. Table 1 shows the content of cellulose, hemicellulose and lignin in some agroindustrial biomass. Small amounts of proteins, pectin and extractives are also found in lignocellulosic biomass.[20]

The rigid and complex structure resulting from the spatial interaction of cellulose, hemicellulose and lignin limits lignocellulosic biomass conversion to the desired product (Fig. 1).

In Brazil, the first efforts carried out by the researchers for the production of 2G ethanol used sugarcane bagasse as a raw material. Sugarcane is an important crop for the Brazilian economy; in 2022/2023, the harvest of sugarcane was 610.1 million metric tons in Brazil, yielding approximately 37.0 million metric tons of sugar and 2.4 billion liters of alcohol.[30] In Brazil, traditional 1G ethanol is obtained from sugarcane by fermentation, which occurs by adding *Saccharomyces cerevisiae* (approximately 10 g L^{-1}) to the sugarcane juice.[6] In the sugar and 1G ethanol production from sugarcane yields a large amount of bagasse (280 kg of bagasse are generated per ton of sugarcane). Most of this biomass is currently burned in sugarcane mills to produce energy, but a significant amount remains, representing great potential for sugar conversion into bio-based products, which defines second-generation (2G) processes.[31] The sugarcane bagasse that arrives at the biorefinery for 2G ethanol production (Fig. 2) contains impurities from the field, so it must first be washed with water. In the next step, washed sugarcane bagasse [1], with initial moisture of 50%, is subjected to pretreatment, to reduce its recalcitrance and to make the polysaccharide

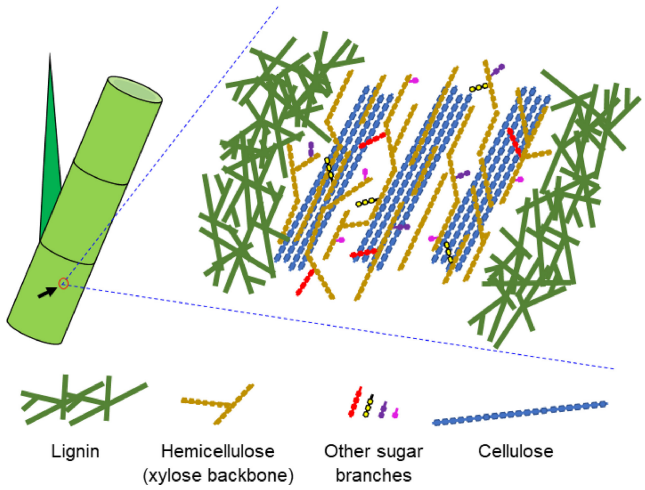

Figure 1. General scheme for the main biomass components (*Source*: Author's own figure).

fraction more accessible to enzymatic action. Several pretreatment [2] methodologies (of a chemical, physical, physicochemical or biological nature) exist, and each has its pros and cons. Effective pretreatment should not result in hemicellulose or cellulose loss or form potential inhibitors of hydrolytic enzymes or microorganisms of ethanol fermentation.[32] Pretreatment accounts for 20–25% of the total production costs and significantly impacts the effectiveness of subsequent unit processes, especially the efficiency with which monosaccharides are released from polysaccharides in the material. The degree of hydrolysis of polysaccharides

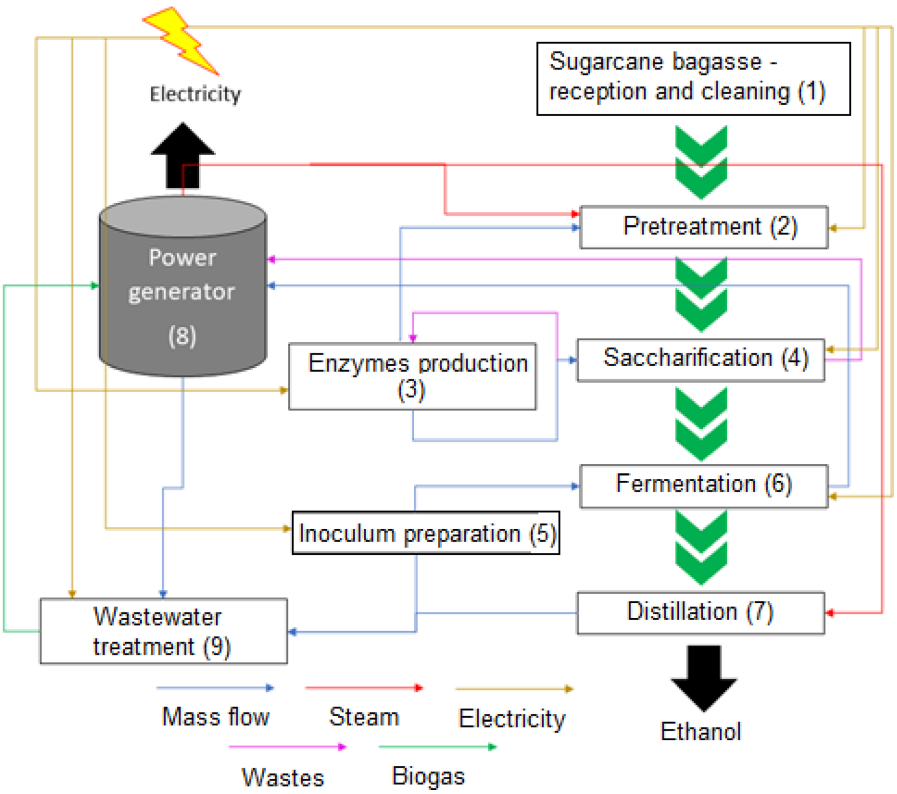

Figure 2. Scheme for the 2G ethanol production plant (*Source*: Adapted from Maga *et al.*[6]).

without pretreatment is lower than 20%, whereas pretreatment increases this degree to 90%.[13,33] Usually, 2G ethanol plant operators prefer to adopt well-consolidated, low-cost strategies such as steam explosion, ammonia fiber explosion and diluted acid hydrolysis on a large scale.

Saccharification [4] is the stage in which polysaccharides from the pretreated lignocellulosic biomass are hydrolyzed to simple sugars by enzyme cocktails [3]. When it comes to using biomass to obtain biofuel and value-added products, efficient enzymatic cellulose and hemicellulose conversion remains a major bottleneck.[11] Some authors suggest that the cost of enzymes for biomass depolymerization should not be more than US$0.10 per liter of ethanol product.[34] Therefore, enzyme cocktails have been produced in the units themselves by microorganisms of the genera *Aspergillus*, *Trichoderma*, *Penicillium* and *Clostridium*. In addition, efforts have been made to produce thermostable cellulases from bacteria (*Bacillus*, *Geobacillus*, *Caldibacillus*, *Acidothermus*, *Caldocellum* and *Clostridium*), fungi (*Chaetomium*, *Talaromyces* and *Thermoascus*) and even the metagenome.[11,35] Regarding Fig. 2, unwanted fractions of the lignin cake obtained during saccharification are sent to the power generator [8]. Part of the obtained broth is used as a culture medium to produce enzymes. On the other hand, the lignocellusic substrate to be fermented [6] may contain a mixture of oligosaccharides, hexoses (glucose, mannose and galactose) and pentoses (xylose and arabinose), and inhibitors such as acids (formic, acetic and levulinic acids), furan derivatives (furfural and 5-hydroxymethylfurfural) and lignin degradation products (vanillin and 4-hydroxybenzaldehyde).[13] Microorganisms that are used to ferment sugars must be able to ferment both C5 and C6 sugars and to resist the presence of inhibitors and ethanol concentration over 40 g dm^{-3}, to obtain ethanol production yields higher than 90%. Additionally, they must be resistant to high temperature and low pH and have low nutritional requirements. Various organisms are available for this step, including some genetically modified organisms; the yeast *S. cerevisiae* is the most popular. However, these yeasts do not have the enzymes that are necessary to ferment pentoses.[36] Numerous bacterial (*Thermus thermophilus* and *Clostridium thermosaccharolyticum*) and yeast (*Pichia stipitis*, *Candida shehatae*, *Pachysolen tannophilus* and *Debaryomyces hansenii*) strains have been reported to employ pentoses, but lower ethanol yields are achieved.[13] Other microorganisms such as *Mucor rouxii* (*Mucor indicus*) can ferment glucose, xylose, mannose, fructose and galactose, and *Scheffersomyces stipitis* can ferment both hexose and pentose sugars. *Mucor*

rouxii shows greater resistance to potential inhibitors present in lignocellulosic hydrolysates, mainly furfural, hydroxymethylfurfural and acetic acid.[37]

The inoculum produced in this stage [5] can be grown in sugarcane molasses containing 60% sugar supplemented with ammonium phosphate. In the end, the generated ethanol is distilled [7] (similarly to 1G ethanol production), and solid wastes are incinerated in the power generator to obtain steam and energy. The generated liquid wastes [9] are treated for water recycling or directed for anaerobic digestion to produce biogas, which in turn is also burned for energy production.[3,6]

Enzymatic saccharification

Enzymatic hydrolysis of lignocellulosic material is assisted by a set of enzymes, commonly classified as carbohydrate active enzymes (CAZymes), which are cataloged in the CAZy database (available at www.cazy.org). They are grouped into five different classes: glycosyl hydrolases (GH), polysaccharide liases (PL), carbohydrate esterases (CE), auxiliary activities (AA) and glycosyl transferases (GT). The first four act on glycan degradation.[38–40] Such classification is based on the similarity of the primary structures, conservation of the secondary and tertiary structures, stereochemical architectures, and catalytic mechanisms.[34] Owing to their prominent role in cellulose and hemicellulose depolymerization, only the enzymes belonging to the GH and AA categories are discussed in more detail in this review.

Cellulose hydrolysis requires several enzymes, such as: endo-1,4-glucanases (EC 3.2.1.4), which hydrolyze amorphous cellulose internal structures; exo-1,4-glucanases (EC 3.2.1.91, also known as cellobiohydrolases), which detach cellobiose molecules from the ends of cellulose; and β-glucosidases (EC 3.2.1.21), which hydrolyze cellobiose to glucose.[40–42] In addition, the recently discovered lytic polysaccharide monooxygenases belonging to families AA9 and AA10 assist in cellulose degradation through an oxidative mechanism.[42,43] The optimum conditions for cellulases to operate include a temperature of 50°C and pH 4.0–5.0.[44] On the other hand, the use of hemicellulases in biomass saccharification is also essential because they increase accessibility to cellulose by solubilizing the hemicellulose structure, which acts as a barrier.[11] Hemicellulose depolymerization requires a more diverse range of CAZymes because this fraction of the biomass comprises a group of heterogeneous polyaccharides.[34,45,46] In the case of xylan, for example, the degradation mechanism involves the hydrolytic enzymes endo-1,4-β-xylanases and β-xylosidases, which catalyze the deconstruction of the main homopolymer chain to D-xylose monomers, and enzymes such as α-L-arabinofuranosidases, α-D-glucuronidases, acetyl xylan esterases, ferulic acid and *p*-coumaric acid esterases, which act to remove the branches.[45] Furthermore, ligninolytic enzymes are important for removing lignin, thereby facilitating enzymatic saccharification of biomass. Laccase, manganese peroxidase and lignin peroxidase are the key enzymes for lignin degradation.[47] Laccases oxidize aromatic amines and phenolic compounds using molecular oxygen as a terminal electron acceptor. Manganese peroxidase and lignin peroxidase can oxidize lignin at the non-phenolic and phenolic aryl-ether positions, respectively.[48] Therefore, complete carbohydrate hydrolysis in pretreated biomass demands a multienzyme complex.

Commercial cellulase and hemicellulase preparations can be obtained from filamentous fungi of the genus Trichoderma (*Trichoderma viride* and *Trichoderma longibrachiatum*), which are considered the most productive and efficient. Novozymes, Genencor and DuPont Industrial Biosciences (DuPont) are the main players: they offer innovative solutions for industrial biorefineries by developing commercial enzymatic preparations that contain cellulase and hemicellulase complexes.[14]

Because the lignocellulosic material has recalcitrant structure, large amounts of enzymes are required to deconstruct it, so the process is costly.[49–52] Thus, the costs of cellulases and enzymatic hydrolysis are the two important constraints in biorefinery commercialization.[14] In general, between 10 and 30 mg g^{-1} cellulase is required for biomass saccharification, which hydrolyzes from 30 to 70% of the cellulose content, to give a hydrolysis rate of 70–90%. The cost of celullase lies between 3 and 8 EUR kg^{-1}, contributing approximately 0.095 EUR kg^{-1} to the hydrolysis cost and 0.18 EUR kg^{-1} to the lignocellulose biomass cost.[53,54] Therefore, enzymatic hydrolysis of biomass remains one of the greatest challenges in biorefineries for 2G ethanol production.

To improve the process yield and hydrolysis rate, research has been geared toward optimizing process conditions and improving enzymatic activities using DoE tools.[50,55,56] Parameters such as enzyme load, pH, temperature, substrate and surfactant concentration, and reaction time govern hydrolysis, so optimizing them is key to enhancing process efficiency.[51,57–61] Besides that, pretreatment optimization can maximize sugar release from sugarcane bagasse because pretreatment makes the biomass more accessible to enzymatic hydrolysis. Thus, DoE tools have been used to optimize the lignocellulosic biomass pre-treatment.[12]

Enzymatic hydrolysis and fermentation can be performed separately (SHF, separate hydrolysis and fermentation) or simultaneously (SSF, simultaneous saccharification and fermentation), or may even entail pre-saccharification

followed by simultaneous saccharification and fermentation (PSSSF, pre-saccharification and simultaneous saccharification and fermentation).[49,62] The advantages and disadvantages of each strategy are detailed in the following topics.

Separated hydrolysis and fermentation

As the name suggests, this type of strategy consists of carrying out hydrolysis and fermentation separately. In the first stage, the pretreated lignocellulosic material is subjected to saccharification mediated by CAZymes, to release glucose and other hexoses/pentoses. Subsequently, the produced monomeric sugars are converted to ethanol by fermentation, which is usually carried out by the yeast species *S. cerevisiae*.[49]

Given that each step occurs separately, they can be performed in their respective ideal operating conditions. Enzymatic hydrolysis is commonly carried out between 45 and 50°C and pH 4.5–5.0.[63] On the other hand, the best ethanol yields are obtained when fermentation occurs at 28–37°C.[49] Despite these positive points, decreased enzymatic activities owing to sugar accumulation in the medium (inhibition by product) and contamination risks are the main disadvantages associated with SHF.[49]

Simultaneous saccharification and fermentation

To overcome the problems involved in SHF, another approach is SSF.[55] As simple sugars are released during hydrolysis, they are consumed and converted to ethanol during fermentation, to give greater ethanol productivity and efficiency.[62] In this way, the rate of enzyme inhibition by the product is reduced, so costs are associated with the large amount of enzymes required in the SHF.[49] Nevertheless, operating under the optimum conditions of both processes is impossible, which constitutes the main limitation of this strategy and makes its implementation difficult.[55,64]

Pre-saccharification and simultaneous saccharification and fermentation

As described previously, SSF was primarily developed to alleviate the problems associated with enzyme inhibition by hydrolysis products. However, carrying out this step under ideal conditions is unfeasible and may result in lower sugar yield compared with SHF.[62] In this sense, the PSSSF principle is to conduct preliminary lignocellulosic material hydrolysis at a temperature that is optimum for enzymatic activity (45–50°C), followed by a decrease in the process temperature to the indirect value for hydrolysis and fermentation (usually optimum for ethanol-producing microorganisms).[65] Thus, PSSSF provides higher sugar yields compared with SSF and higher ethanol productivity compared with SHF.[62]

Application of DoE to optimize enzymatic saccharification

As previously mentioned, enzymatic saccharification is the critical step for releasing monomeric sugars during 2G ethanol production and constitutes a challenge for researchers attempting to make bioethanol production more economically viable.[58,66,67] The performance of this stage is governed by numerous parameters, so optimizing them is key to increasing process efficiency.[57] Fig. 3 illustrates the saccharification process, the several factors (independent variables) that could affect this process in 2G ethanol refineries, and the most important responses (dependent variables) to be analyzed in the final broth. For simplification, the biomass is represented only by deconstructed cellulose and xylose (main hemicellulose component) fibers. The represented cellulases are endoglucanases (red), cellobiohydrolases (yellow) and β-glucosidases (light green), and the represented hemicellulases are endoxylanases (pink), exoxylanases (orange) and β-xylosidases (dark green). In purple, lytic polysaccharide monooxygenase represents the only redox enzyme acting on biomass along with the other hydrolytic enzymes. In this representation, some enzymes are attached to carbohydrate binding molecules by linker regions.

To study how factors or independent variables affect the dependent variables shown in Fig. 3, some researches have used DoE tools for determining the simultaneous or individual influence of all the factors involved in the process using a minimum number of experiments, aiming at an optimized scenario.[62] Briefly, the application of any experimental design involves the following steps: (1) stipulating experimental limits; (2) defining specific experimental conditions; and (3) using mathematical analysis to predict the response at any point within the pre-stipulated experimental limits.[68]

Design of experiments generally involves two main strategies. The first strategy encompasses the use of screening experiments to identify and to select the factors that significantly affect the responses, which is then followed by optimization. Screening design tools to select factors includes

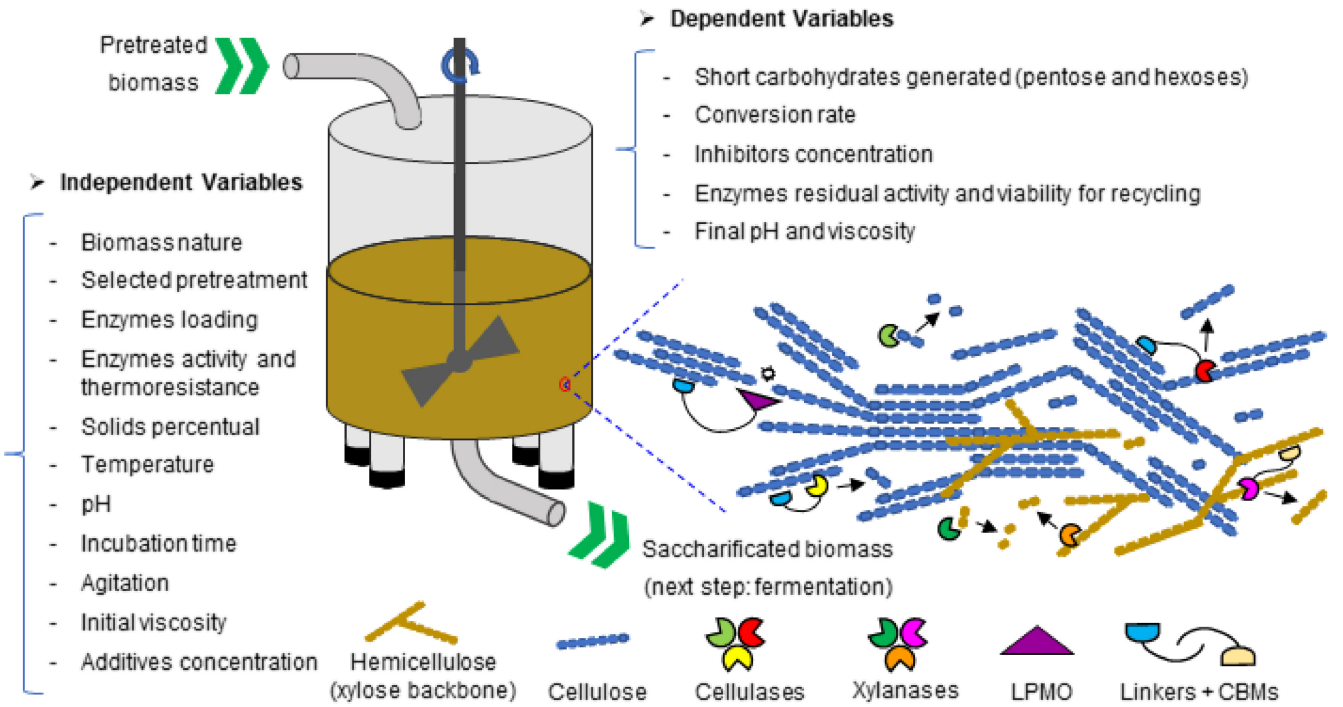

Figure 3. Saccharification step variables in 2G ethanol refineries and illustration of the enzymatic action on pretreated biomass (*Source*: Author's own figure).

full or fractional factorial and Plackett–Burman designs. On the other hand, the second strategy is to perform direct optimization of the factors that the researcher considers significant. Central composite design, Box–Behnken design and RSM can be used to optimize the process.[69]

Table 2 lists some of the main experimental design tools found in the literature for optimizing enzymatic saccharification.

Screening design for selecting factors

Screening experiments are applied as a preliminary step before optimization itself to select the factors and their levels that significantly affect the desired response. The most commonly used tools include fractional factorial and Plackett–Burman designs.[56,69]

Fractional factorial design

Experiments outlined in full factorial schemes involve all the possible interactions between levels of two or more factors. In the case of a two-level FFD, a number of experiments correspond to 2^k, with k being the number of factors involved; each factor has a high (represented by +1) and a low (represented by −1) value.[69,77] However, if the interactions between the studied parameters are believed to be negligible, or if the overall number of experiments is simply too high (e.g. the two-level FFD for seven tested factors constitutes 128 experiments), FFD might become unnecessary. In such case, FFD is a finite fraction ($1/xr$) of a complete or 'full' FD, where r is the degree of fractionation, and $xk - r$ is the total number of required experiments. This type of design experimental is more suitable when $k > 4$.

Noratiqah *et al.*[70] applied a 2^5 FFD to detect the factors that most impacted on concentration of reducing sugars from enzymatic OPEFB (oil palm empty fruit bunch) biomass degradation. The authors evaluated the effects of the five independent variables – OPEFB concentration, temperature, incubation time, Tween 80 concentration and agitation speed – under 37 experiments (including five central points). All of the evaluated independent variables significantly affected hydrolysis in the stipulated confidence interval ($\alpha = 0.05$), so they were selected for the subsequent step involving optimization of the parameters through CCD.

Plackett–Burman design

The statistical design tool developed by Plackett and Burman corresponds to a fractional factorial of two levels (−1, +1) that involves constructing Hadamard matrices.[77] Such orthogonal

Table 2. Design of experiments applied in to optimize enzymatic saccharification of different biomasses.

Experimental design	Biomass	Factors or independent variables	Optimum conditions	Responses or dependent variables	Optimum response predicted	Reference
Plackett–Burman and CCD	Rock-rose (*Cistus ladanifer*)	Temperature (°C)	50	Glucose production ($g\,g^{-1}$ of biomass)	0.326	56
		pH	4.86			
		Cellulase (FPU g^{-1})	60			
		PEG 4000 ($g\,g^{-1}$)	0.4			
		Incubation time (h)	72			
	Broom (*Cytisus striatus*)	Temperature (°C)	50		0.427	
		pH	4.53			
		Cellulase (FPU g^{-1})	60			
		PEG 4000 ($g\,g^{-1}$)	0.27			
		Incubation time (h)	72			
Full factorial and CCD	OPEFB	OPEFB concentration (% g m^{-3})	1.95	Reducing sugar concentration ($g\,L^{-1}$)	1.171	70
		Temperature (°C)	55			
		Incubation time	3 days and 16 h			
		Tween-80 concentration (%)	0.5			
		Stirring speed (rpm)	87.5			
CCD	Rice straw	Enzyme concentration (FPU g^{-1})	37.5	Saccharification yield (%)	88.9	71
		Substrate concentration (%)	2.4			
		Temperature (°C)	35.4			
		pH	5.2			
CCD	Poplar wood	Enzyme concentration (FPU g^{-1})	65	Reducing sugar concentration ($g\,L^{-1}$)	29.8	66
		Substrate concentration (%)	10			
		Temperature (°C)	40			
		pH	—			
CCD	*Parthenium* sp.	Temperature (°C)	50	Saccharification yield (%)	80.08	50
		pH	4.53			
		Enzyme loading (mL)	0.8			
		Amount of substrate (g)	0.24			
CCD	Corn straw	Novozyme 188 ($\mu g\,g^{-1}$)	377	Saccharification yield (%)	45.8%	72
		Pectinase ($\mu g\,g^{-1}$)	171			
		Sodium thiosulfate ($mg\,mL^{-1}$)	1			
CCD	SSB	Substrate concentration (%)	15	Glucose concentration ($g\,mL^{-1}$)	68.58	73
		Incubation time (h)	58			
		Celluclast ($IU\,mL^{-1}$)	20			
		Temperature (°C)	60			
CCD	Cane straw	Enzyme loading (FPU g^{-1})	14.5	Ethanol yield (%) and productivity ($g\,L^{-1}\,h^{-1}$)	70.63 and 0.74	62
		Biomass concentration (% g m^{-3})	19.3			
		Pre-saccharification time (h)	33			
CCD	Kans grass	Solid loading (%, w v^{-1})	24	Ethanol concentration (%, v/v), productivity ($g\,L^{-1}\,h^{-1}$), ethanol conversion efficiency (%), and yield ($g\,g^{-1}$).	7.62, 2.50, 65.01, 0.249	74
		Incubation time (h)	24			
		Temperature (°C)	37			
		Inoculum volume (%, v v^{-1})	8.8			
		Laccase to cellulase ratio	1:6			

Table 2. (Continued).

Experimental design	Biomass	Factors or independent variables		Optimum conditions	Responses or dependent variables	Optimum response predicted	Reference
Box–Behnken	Rice straw	Temperature (°C)		51.45	Hydrolysis yield (%)	1.42	75
		Reaction time (h)		3.84			
		pH		—			
		Enzyme loading (mL)		—			
Box–Behnken	Water hyacinth biomass	Substrate concentration (% g g^{-1})		9.92	Total of reducing sugars (g g^{-1})	0.5447	60
		Cellulase (U g^{-1})		49.56			
		Xylanase (U g^{-1})		280.33			
		Surfactant concentration (% g g^{-1})		0.13			
Box–Behnken	Potato peel residue	Temperature (°C)		45	Reducing sugars (g L^{-1})	77.1	57
		pH		5.0			
		Substrate concentration (% g m^{-3})		10			
		Tween-80 concentration (%)		0.5			
Box–Behnken	*Bombax seiba* seed pods	Substrate loading (%)		10	Cellulose content (%), total sugars (mg mL^{-1}), reducing sugars (mg mL^{-1}) and total phenol (mg mL^{-1}) released	60, 233.7, 17.8 and 128.1	76
		NaOH concentration (%)		5			
		Pretreatment time (h)		4			
		Substrate loading (%)		15		54, 259.5, 53.5 and 119.3	
		NaOH concentration (%)		5			
		Pretreatment time (h)		6			
		Substrate loading (%)		15		36, 169.1, 87.4 and 204.6	
		NaOH concentration (%)		1			
		Pretreatment time (h)		6			
		Substrate loading (%)		15		54, 223.6, 59.9 and 246.8	
		NaOH concentration (%)		3			
		Pretreatment time (h)		4			
Box–Behnken	Corncob	PSSSF	Yeast (cells mL^{-1})	5.40×10^6	Ethanol concentration (g L^{-1}) and conversion (%)	41.78 and 70.52	64
			Amount of biomass (% g m^{-3})	17.5			
			Enzyme loading (FPU g^{-1})	30			
		SSF	Yeast (cells mL^{-1})	2.70×10^6		39.99 and 66.46	
			Amount of biomass (% g m^{-3})	17.82			
			Enzyme loading (FPU g^{-1})	30			

CCD, central composite design; OPEFB, oil palm empty fruit bunch; PSSSF, pre-saccharification and simultaneous saccharification and fermentation; SSF, simultaneous saccharification and fermentation; SSB, sweet sorghum bagasse. One FPU of enzyme is defined as the amount of enzyme catalyzing the release of 1 μmol of glucose equivalent per min.

matrices are constructed by considering the maximum number of factors (columns) to be examined as N–1 in a total of N performed experiments (N is defined as a multiple of 4).[77,78] This type of design does not consider any interactions between the factors, so it is only useful for estimating the main effect of the factors involved in the process and cannot be used to obtain surface response during optimization of said process.[53] Therefore, the PB design constitutes an important tool to select variables and is only an intermediate step that guides and leads to the final design.[53,59,61]

Ferreira *et al.*[56] used PB to identify the factors that significantly affected the enzymatic hydrolysis of the lignocellulosic substrates *Cistus ladanifer* (rock rose) and *Cytisus striatus* (broom). The authors screened 11 variables (pH, temperature, buffer, buffer concentration, cellulase concentration, β-glucosidase concentration, substrate concentration, reaction volume, incubation time, agitation and PEG 4000 concentration) through 12 experimental runs, to find that five of these variables – pH, temperature, cellulase concentration, incubation time and PEG4000

concentration – significantly and positively affected the hydrolysis of at least one of the substrates. Among these variables, cellulase concentration was the most significant and positively impacted both responses. After screening, the next step was to determine the optimum values of the five factors selected for study by using CCD to generate the response surface.

Response surface methodology as a tool for optimization

After screening experiments are accomplished, the selected factors can be optimized through the RSM: a set of statistical and mathematical techniques developed by Box and Wilson in the 1950s.[69] Response surface methodology plays an essential role in visualizing the relationship between independent (factors) and dependent variables (response) through second-order mathematic models that include the linear, interaction and quadratic effects (Eqn 1).[57,79]

$$y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{\substack{i=1 \\ i=1}}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

where y is the predicted mean response, β_o is the constant term of the model, β_i are the coefficients of the linear terms, β_{ii} are the coefficients of the quadratic terms, β_{ij} are the coefficients of the interaction terms and ε is the random error component that is determined by fitting the model to the data.

This second-order polynomial model is reasonably flexible and accurately describes the curvature and interactions, which makes it a suitable model to optimize the process and to infer nontrivial phenomena.[50,56,72] The mathematical model can be considered adequate and predictive when the regression is statically significant ($F_{\text{Regression}} >> \text{Ftab}$, P-value $< \alpha$) mathematic model) and does not present an error ($F_{\text{lack of fit}} < \text{Ftab}$, P-value $> \alpha$, lack of fit is not significant). The significance level, also called α, can be 0.05 or 0.1. Besides that, the coefficient of determination (R^2) and the adjusted coefficient of determination (R_{adj}^2) represent the percentage of variance explained by the model, and these coefficients should be analyzed. Thus, the response surface can be obtained with a significant regression and can be used to find the maximum region (Fig. 4).[80] Other profiles for the quadratic response surface plot in the optimization of two variables can be obtained, such as surfaces with a plateau in relation to one variable, the surface showing the maximum point outside the experimental region, the surface with a minimum point and the surface with a saddle point as the critical point.[81]

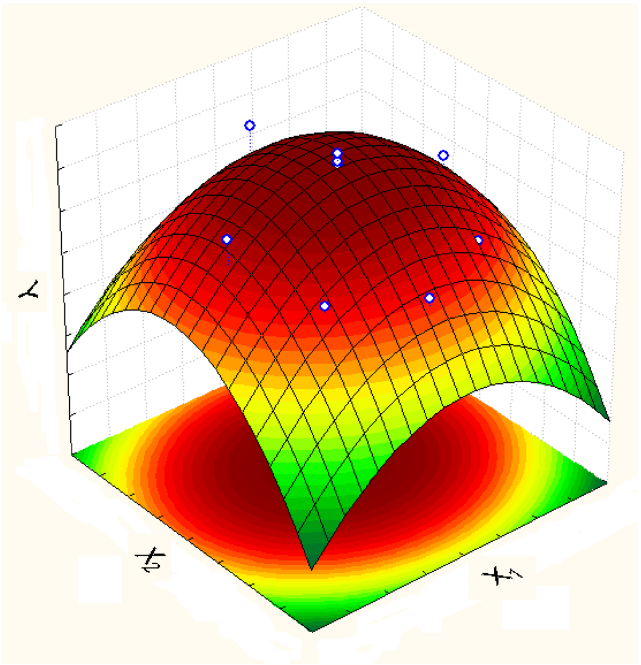

Figure 4. Surface response with a maximum region generated from a quadratic model in the optimization of two variables (*Source*: Author's own figure).

When a large number of responses must be optimized, the desirability function is the most popular strategy to be performed.[82] The combined application of RSM and the desirability approach, called the 'desirability optimization methodology', represents an efficient tool even when it involves antagonistic responses (multi-objective optimization).[62,83] The desirability optimization methodology initially involves transforming each estimated response (y_i) into a $d(y_i)$ function, called desirability, which ranges from 0 (undesirable response) to 1 (most desirable response). Then, an overall desirability function (D) is calculated as a geometric average of all the individual desirability functions (d), as shown in Eqn (2):

$$D = \left(d_1 \times d_2 \times d_3 \times \ldots \times d_n\right)^{\frac{1}{n}} \quad (2)$$

where n denotes the number of responses.

In general, the desirability function allows multivariate optimization problems to be condensed into univariate ones, in which the optimum solutions are determined by maximizing the D value with respect to the independent factors.[83,84]

The desirability function for a nominal the best type response is defined as follows (Eqn 3):

$$d_i(\hat{y}_i(x)) = \begin{cases} 0 & \text{if } \hat{y}_i(x) \ll Y_i^{min} \text{ or } \hat{y}_i(x) > Y_i^{max}, \\ \left[\dfrac{\hat{y}_i(x) - Y_i^{min}}{T_i^{min} - Y_i^{min}}\right]^{s_i} & \text{if } Y_i^{min} < \hat{y}_i(x) \ll T_i^{min}, \\ \left[\dfrac{Y_i^{max} - \hat{y}_i(x)}{Y_i^{max} - T_i^{max}}\right]^{t_i} & \text{if } T_i^{max} < \hat{y}_i(x) \ll Y_i^{max}, \\ 1 & \text{if } T_i^{min} < \hat{y}_i(x) \ll T_i^{max}, \end{cases} \quad (3)$$

where $d_i(y_i(x))$ is the desirability function of $y_i(x)$; Y_i^{min} and Y_i^{max} are the lower and upper bounds on the response, respectively; T_i^{min} and T_i^{max} ($T_i^{min} \leq T_i^{max}$) are the lower and upper targets of the response, respectively; and s_i and t_i are the parameters that determine the shape of $d_i(y_i(x))$. If s_i (or t_i) = 1, the shape is linear; if s_i (or t_i) > 1, the shape is convex; and if $0 < s_i$ (or t_i) < 1, the shape is concave.[85]

Some studies have shown that RSM can be successfully applied to optimize the enzymatic hydrolysis of numerous substrates.[56,57] Among the most used designs in RSM, the CCD and the BBD stand out.[56] The topics below detail these designs and provide some works that have used these tools to optimize saccharification.

Central composite design

Central composite design is the most commonly used experimental design tool in RSM for fitted quadratic models.[56,69] It is usually based on a two-level factorial design, with the addition of 2*k* axial points (where *k* is the number of factors), which represent the two new extreme values of each factor and an arbitrary number of repetitions at the central point.[68] The addition of axial points is precisely the main difference between CCD and FFD, which enables the quadratic terms to be estimated.[69] The axial points of a CCD are defined as $\pm\alpha$ [where $\alpha = (2k)^{1/4}$].[77] A CCD generally involves a number of *N* experiments corresponding to 2^k factorial points + 2k axial points + *n* central points ('*n*' being an arbitrary number).[77]

Central composite design has been extensively applied to find the optimum conditions for the enzymatic hydrolysis of different lignocellulosic biomasses. Jeya *et al.*[71] used CCD to optimize the saccharification of alkali-treated rice straw by RSM. The authors randomly conducted 30 experiments (including eight axial points and six replications in the central point) to investigate how the parameters *A* (enzyme concentration, 7.5–37.5 filter paper units (FPU) g^{-1}), *B* (substrate concentration, 0.75–3.75%), *C* (temperature, 15–55°C) and *D* (pH, 3–7) affected saccharification (%). They developed a second-order regression model using the experimental results. ANOVA revealed a high coefficient of determination ($R^2 = 0.9837$) for the model, which proved that the experimental and predicted results agreed. The authors found that optimum saccharification (88.9%) could be obtained at 35.4°C and pH 5.2, with 2.4% substrate and 37.5 FPU g^{-1} of substrate. A subsequent experiment under the specified conditions allowed obtaining a yield of 88% (685 mg g^{-1} of substrate) in the biomass saccharification.

Similarly, Jeya *et al.*[66] used CCD to determine the influence of enzyme and substrate concentrations, temperature and pH and to optimize these factors, aiming at maximum sugar production from poplar wood biomass hydrolysis. The *F*-test results showed that three linear terms, four quadratic terms and four interaction terms were significant. The maximum response (29.8 g L^{-1}) with an enzyme level of 65 FPU g^{-1}, 10% of substrate and 40°C was predicted. Experimental validation of optimized conditions resulted in total reducing sugar production of 29.3 g L^{-1} (293 mg g^{-1} of substrate), so the model was considered valid and useful for predictions.

Pandiyan *et al.*[50] optimized enzymatic saccharification of pretreated *Parthenium* sp. biomass through 2^4 CCD. By carrying out 30 experiments (2^4 factorial points + 2 × 4 axial points + 6 repetitions in the central point), the authors investigated how *A* (temperature, 45–65°C), *B* (pH, 4–5), *C* (enzyme load, 0.2–1 mL) and *D* (substrate concentration, 0.1–0.5 g) and the interactions between them affected the saccharification yield (%). They obtained a second-order polynomial equation describing the relationship between the independent variables from the experimental results; the coefficient of determination (R^2) was 0.96, indicating good fit of the model. According to ANOVA, the terms *A*, *C*, *D*, *AD*, A^2, B^2 and C^2 significantly influenced the response ('*P* value' < 0.05). These terms were considered in the mathematical model, which was used to predict the maximum sugar production yield (corresponding to 80.08%). To obtain this yield, the biomass saccharification should be performed in the following conditions: pH 4.53, 50°C, 0.8 mL of enzymes (7 FPU g^{-1}) and 0.24 g of substrate. A triplicate experiment conducted under the best conditions resulted in 85.8% (574 mg per gram dry substrate (mg gds^{-1}) hydrolysis yield, which was considered to agree well with the predicted value, thereby validating the mathematic model.

Yu *et al.*[72] also applied CCD to determine the optimum values of the factors X_1 (ovozyme 188, μg g^{-1}), X_2 (pectinase, μg g^{-1}) and X_3 (sodium thiosulfate, mg mL^{-1}) to maximize glucose production from corn straw. Both the coefficients of the quadratic and linear of X_1 ($P < 0.01$) and the coefficient

of the X_2 quadratic ($P < 0.05$) were significant. From the generated 2D contour graphs, the authors determined the optimum values of the three variables and understood the interactions between them. The analyses indicated that a maximum yield (45.8%) could be reached using 377 $\mu g\,g^{-1}$ of Novozyme 188, 171 $\mu g\,g^{-1}$ of pectinase and 1 $mg\,mL^{-1}$ of thiosulfate. When the experiment was carried out in the optimum conditions, the maximum response was 44.9%, which was consistent with the predicted result, thus validating the statistical analysis.

Lavudi *et al.*[73] used CCD to optimize both the alkaline pretreatment and enzymatic hydrolysis steps of sweet sorghum bagasse (SSB), to maximize the concentration of released sugars (glucose and xylose) and ethanol production from SHF and SSF. Regarding hydrolysis, the authors selected four factors: *A* (substrate concentration, 7.5–17.5%), *B* (incubation time, 6–78 h), *C* (concentration of Celluclast® cocktail, 5–25 IU mL^{-1}) and *D* (temperature, 30–70°C) for optimization. The design matrix consisted of 27 experiments. ANOVA indicated that the linear terms of *A*–*D* were substantially more pronounced than the other terms, so these were the most significant factors influencing enzyme saccharification of pretreated SSB. The mathematical model for glucose was statistically significant but it has lack of fit ($P < 0.05$). However, the authors considered that the high value of R^2 value (0.9066) indicated that the model was suitable to predict enzymatic hydrolysis of pretreated SSB. On the other hand, the mathematical model obtained for xylose was statistically significant and predictive (lack of fit was not significant). By analyzing the generated response surfaces for glucose and xylose, the authors found that they could achieve the optimum response by applying 15% substrate, incubation for 58 h, 20 IU mL^{-1} of cellulases and 60°C during hydrolysis. Using the optimal conditions of enzymatic hydrolysis of pretreated SSB glucose, xylose, arabinose and cellobiose were obtained at concentrations of 53.02, 14.70, 2.10 and 0.97 $g\,L^{-1}$, respectively.

Likewise, Pratto *et al.*[62] sought to optimize pre-saccharification (PS) of hydrothermally pre-treated cane straw to increase the ethanol yield (%) and productivity ($g\,L^{-1}\,h^{-1}$) of PSSSF. The authors assessed how the factors X_1 (enzymatic dosage, 5–15 FPU g^{-1} cellulose), X_2 (biomass concentration, 5–25% g m^{-3}) and X_3 (pre-saccharification time, 6–42 h) influenced the response variables using CCD based on 20 experimental results (2^3 factorial points + 2×3 axial points + 6 repetitions at the central point). The results were statically analyzed and a quadratic polynomial mathematic model was obtained presenting significance and without lack of fit, which ensured that the models were able to predict results within 95% confidence interval. To interpret the results better, the authors constructed 3D response surface graphs from the quadratic mathematical models. The authors achieved the highest ethanol yield (73.6%) when they applied 13 FPU g^{-1} of enzyme, 9.05% of biomass and reaction for 34.7 h during PS. On the other hand, the conditions of optimum productivity ($0.92\,g\,L^{-1}\,h^{-1}$) were 13 FPU g^{-1} of enzyme, 20.95% of biomass and reaction for 13.3 h. Then, to maximize both response variables simultaneously, the authors employed a multiresponse optimization method that used the overall desirability function (*D*). Thus, the authors found that PSSSF reactor operation with 14.5 FPU g^{-1} of enzyme, 19.3% of biomass and pre-saccharification for 33 h provided the optimum values of ethanol yield (70.63 ± 5.6%) and productivity ($0.74 \pm 0.05\,g\,L^{-1}\,h^{-1}$).

In other work, the ethanol production process from raw Kan grass was optimized by RSM, using a 2^5 full factorial CCD. However, the authors performed only 32 experiments (2^5), while the number of experiments to perform a centered composite design 2^5 should be 48 ($2^5 + 2 \times 5 + 6$ central points). The total number of experiments was lower because the authors used a fractional design 2^{5-1} (16 experiments) and not full factorial design ($2^5 = 32$ experiments). Moreover, the authors reduced the number of levels of factors from 5 ($-\alpha$, −1, 0, +1, $+\alpha$) to 3 (−1, 0, +1) and applied −1 and + 1 as axial points. The factors (and their levels) were solid loading (15–25%, w v^{-1}), incubation time (12–36 h), temperature (35–40°C), inoculum volume (8–12%, v v^{-1}) and laccase to cellulase ratio (1:4–1:8). The activities of laccase (3125 IU g^{-1}) and cellulase–xylanase had been previously studied and were maintained at 3125 and 75 IU g^{-1}, respectively. The authors analyzed the response surfaces of laccase to cellulase ratio vs. temperature, temperature vs. inoculum volume, and laccase to cellulase ratio vs. solid loading for ethanol generation and concluded that the optimized conditions for Kans grass bioprocessing were solid loading of 24% (w v^{-1}), an incubation time of 24 h, a temperature of 37°C, an inoculum volume of 8.8% (v v^{-1}) and a laccase to cellulase ratio of 1:6. They did not use the multiresponse analysis methodology to find the optimal conditions for bioethanol production. By using these optimal conditions, 7.62% of ethanol was obtained, which was close to the predicted ethanol yield of 8.10% (v/v).[74]

Alkaline pretreatment for increased sugarcane bagasse saccharification was optimized by high-resolution FFD combined with a central composite orthogonal design. A total of 46 experimental conditions were evaluated, and the maximum sugar yield was determined. This robust DoE resulted in maximum enzymatic sugarcane bagasse hydrolysis

efficiency and further indicated that this combined approach is versatile for other lignocellulosic biomasses.[86]

Box–Behnken design

As well as CCD, BBD is commonly used in RSM. Box–Behnken design is a second-order rotational design in which only three equidistant levels (high, medium and low) will be defined for each independent variable.[68,69] The number of N experiments required to develop a BBD can be calculated as $2k(k-1)+n$ central points (with $3 \leq n \leq 5$). Box–Behnken design is generally applied as an alternative to three-level factorial design because it requires a reduced number of experiments while producing satisfactory results.[69]

Chen *et al.*[75] applied BBD to increase the hydrolysis efficiency of corn and rice straw by GH5 endoglucanase from *Aspergillus glaucus*, called AgCMCase. The authors carried out 29 experiments to obtain the optimal values of the parameters X_1 (temperature, 50–70°C), X_2 (reaction time, 2–4 h), X_3 (pH, 5.0–7.0) and X_4 (enzyme dosage, 1–2 mL). They assessed the regression coefficients and the significance of each variable and determined their interactions. The analyses revealed that the linear coefficients X_2 and X_4, the quadratic coefficients ${X_1}^2$ and ${X_2}^2$, and the interactions between X_1 and X_2 were significant for the hydrolysis of both substrates. Three-dimensional response surface plots were able to estimate the optimum conditions to produce reducing sugars. According to the prediction data, the maximum yield (1.42% for rice straw) could be reached at medium temperatures in an appropriate reaction time (51.45°C and 3.84 h for rice straw, respectively). The validation experiments resulted in 1.61% released sugars when the aforementioned optimum conditions were used in the reactions.

Similarly, Das *et al.*[59] used BBD to estimate the optimum conditions for the enzymatic process, aiming to maximize reducing sugar production from water hyacinth biomass. The parameters A (substrate concentration, 4–10% g g^{-1}), B (cellulase, 20–50 U g^{-1}), C (xylanase, 150–300 U g^{-1}) and (D, surfactant 0.1–0.2% g g^{-1}) significantly affected the response variable in the stipulated confidence interval ($P<0.05$), so the authors optimized them by RSM (29 experiments, including five central points). The significant and non-significant values for model regression and lack of fit, respectively, attested that the quadratic model was valid and predictive. Thus, the authors generated and analyzed the response surfaces and implemented a desirability function to increase the accuracy of the optimum experimental values for the reaction. They were able to determine that the maximum yield (0.5447 g g^{-1}) could be achieved when operation conditions included 9.92% (g g^{-1}) substrate, 49.56 U g^{-1} cellulase, 280.33 U g^{-1} xylanase and 0.13% (g g^{-1}) Tween-80. This statistical forecast was experimentally validated, and a very similar yield was obtained, equivalent to 0.5524 g g^{-1}.

Taher *et al.*[57] adopted the same design tool to evaluate how V_1 (temperature, 30–60°C), V_2 (pH, 5–8), V_3 (substrate concentration, 2–10% g m^{-3}) and V_4 (Tween-80 concentration, 0–1%) influenced on the yield of potato peel residue hydrolysis. After analyzing the generated response surfaces, the authors were able to predict that the release of reducing sugars from biomass was maximum (77.1 g L^{-1} or 84%) when they carried out the reaction in the presence of 10 and 0.5% substrate and surfactant, respectively, at 45°C and pH 5.0. They verified that the model was valid by conducting three experiments with different combinations of variable values within the experimental range stipulated in the design. When they compared the observed values with the predicted ones, they confirmed that the method was valid.

Sewsynker-Sukai and Kana[64] also applied BBD to develop SSF with (PSSSF) and without pre-saccharification, to produce bioethanol from pre-treated corncob. The authors optimized the studied parameters A (yeast, 2.7×10^6–1.35×10^7 cells mL^{-1}), B (amount of biomass, 10–30% g m^{-3}) and C (enzyme load, 10–30 FPU g^{-1}) to maximize ethanol concentration and conversion. They accomplished 17 experiments (including five central points) for each configuration (PSSSF and SSF) and obtained two mathematic models, corresponding to the two evaluated responses. For PSSSF, they estimated that maximum ethanol concentration (41.78 g L^{-1}) and conversion (70.52%) could be reached using 5.40×10^6 yeast cells mL^{-1}, 17.5% substrate and 30 FPU g^{-1} enzyme. For SSF performed without previous hydrolysis, they obtained similar results. When they used the same substrate and enzyme concentrations in addition to 2.70×10^6 yeast cells m L^{-1}, the predicted optimum responses were 39.99 g L^{-1} and 66.46%, respectively. Such predictions agreed with the maximum responses observed in the experimental validation for PSSSF (36.92 ± 1.34 g L^{-1} and 62.36 ± 2.27%) and SSF (35.04 ± 0.170 g L^{-1} and 58.13 ± 0.283%).

In other work, the BBD and RSM were used to optimize *Bombax ceiba* waste pretreatment.[76] The optimal conditions allowed to obtain biomass with high cellulose content (60%). These conditions were: 10% substrate loading, 5% NaOH concentration, and 4 h residence time. The pretreated biomass was subjected to saccharification and fermentation using SHF and SSF with the commercial enzyme and *S. cerevisae*. The authors observed that SSF produced fermentable sugars (50.9% after 24 h) and bioethanol (54.51 g L^{-1}after 96 h) more efficiently.

Optimization of enzymatic potato peel hydrolysis included a pretreatment step followed by enzymatic hydrolysis.

Pretreatment optimization by experimental design is also essential for maximizing biomass hydrolysis because it can improve the conditions for liquefaction, enzymatic hydrolysis and fermentation. A study found that eight experiments were enough to show the importance of optimizing enzyme loading by BBD. Three independent variables at three levels were able to determine the load Viscozyme and San super load that provided the highest ethanol production ($117\,g\,L^{-1}$).[87]

Comparison between the optimized and non-optimized processes

On the basis of the previous discussion about the results obtained by some authors after they applied DoE tools together with RSM to optimize enzymatic lignocellulosic biomass hydrolysis, we compared the yields of statistically optimized and non-optimized saccharification. Table 3 summarizes such comparisons.

As expected, optimizations by RSM improved process performance. Noratiqah *et al.*[70] reported a 1.07-fold higher concentration of reducing sugars from OPEFB hydrolysis after optimization with CCD. In another study, Jeya *et al.*[66] obtained a 1.63-fold improvement in reducing sugar production from poplar wood biomass saccharification. Similarly, sugar yields from rice straw saccharification increased 1.25[71] and 1.52[75] fold after statistical optimization of the operational parameters by CCD and BBD, respectively. Likewise, optimizing potato peel residue hydrolysis by BBD yielded 1.3 times more sugars.[57] Finally, Pandiyan *et al.*[50] reported that *Parthenium sp*. saccharification under the optimized conditions resulted in 1.1-fold higher efficiency compared with the non-optimized process.

Song *et al.*[88] optimized enzymatic hydrolysis of the pre-treated rice straw to obtain maximum saccharification yield. This statistical optimization by RSM increased saccharification by 3.4-fold using parameters demonstrating that the model was significant and efficient for lignocellulosic biomass bioconversion to fermentable sugars on an industrial scale.

Conclusions and future perspectives

This review has shown that different experimental design tools (DoE) have been successful in optimizing biomass saccharification processes for production of bioethanol. We retrieved 134 publications in the period spanning from 2012 to 2023 – according to Web of Science data (https://apps.webofknowledge.com/) on 2 March 2023 (keywords: experimental design, saccharification and bioethanol). Some were analyzed in this review and allowed experimental design strategies used by researchers to be detailed. We consider that initially researchers should have knowledge about the relevant independent variables or factors for overall enzymatic saccharification of specific biomass in order to successfully apply experimental designs and RSM to process optimization. We have identified that the main factors studied by the authors included pretreatment conditions, temperature, pH, reaction time and concentrations of enzymes, substrates and surfactants. The values of these factors could be defined using preliminary tests or based on previous studies found in the literature. We have observed that few authors used screening methods to evaluate the effect of factors and select the most relevant factors and subsequently carry on the optimization study. Meanwhile most authors used CCD and BBD to generate response surfaces and obtain the optimum saccharification parameters. In addition, RSM was used as tool to obtain the response surface, which was analyzed to determine the maximum region that allowed the optimum conditions of the process to be found. All of the studies had the predicted and experimental values agreed satisfactorily. On the other hand, we observed that few studies used the multiresponse methodology to obtain the optimal condition to biomass

Table 3. Comparison between optimized and non-optimized saccharification.

Biomass	Reducing sugars		References
	Non-optimized process	Optimized process	
OPEFB	$1.108\,g\,L^{-1}$	$1.183\,g\,L^{-1}$	70
Poplar wood	$180\,mg\,g^{-1}$ substrate	$293\,mg\,g^{-1}$ substrate	66
Rice straw	$546\,mg\,g^{-1}$ substrate	$685\,mg\,g^{-1}$ substrate	71
Rice straw	1.06%	1.61%	75
Parthenium sp.	$513\,mg\,gds^{-1}$	$574\,mg\,gds^{-1}$	50
Potato peel residue	$57.8\,g\,L^{-1}$	$77.1\,g\,L^{-1}$	57

saccharification process. We believe that this methodology can help to determine an optimal condition that satisfies different responses requirements.

Using the optimal conditions obtained by DoE tools and RSM, greater hydrolysis yields and productivity compared with the non-optimized process were achieved, corroborating that using DoE and RSM is an efficient statistical approach for optimizing saccharification. Regarding enzyme concentration, greater amounts of biocatalyst do not necessarily imply greater substrate conversion. Whereas the cost of enzymes is currently one of the main challenges for achieving economically viable production of 2G ethanol (among other bioproducts) in lignocellulosic biorefineries, an extra advantage of applying DoE in the hydrolysis step is to minimize the necessary amount of enzymes.

Finally, on the basis of the results and discussions presented here, we can make the following considerations: when DoE is correctly applied, it represents an essential approach for planning any research because it not only optimizes the target response(s) of a process, but also contributes to reducing the costs and time spent on carrying out the experiments. However, improper use of DoE tools can lead to wrong results. In this context, screening design cannot be used to optimize the process because a reduction in the number of experiments affects the design resolution and causes important information to be lost.

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Conflicts of interest

The authors declare no conflicts of interest.

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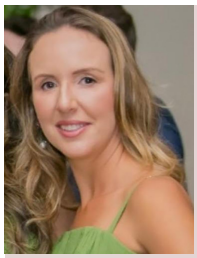

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